POSSIBLE OVERWINTERING RESERVOIRS AND EXPERIMENTAL EPIDEMIOLOGY OF WESTERN EQUINE ENCEPHALITIS VIRUS

by

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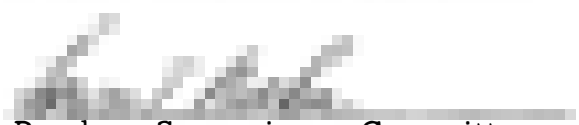

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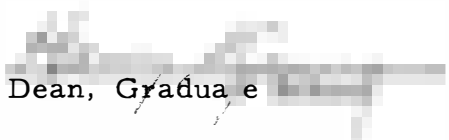

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POSSIBLE OVERWINTERING RESERVOIRS AND EXPERIMENTAL EPIDEMIOLOGY OF WESTERN EQUINE ENCEPHALITIS VIRUS

INTRODUCTION

In previous studies reported from this laboratory, data have been collected which indicate the importance of reduced temperatures in maintaining Western equine encephalitis (WEE) virus in hibernating infected snakes (Gebhardt and Hill, 1960) and in chick embryo cells infected in vitro and incubated at 25° C (Dolana, 1961) Since then further attempts have been made both in the laboratory and in the field to find the significant overwintering reservoir or reservoirs for WEE virus in an area, such as Utah, where a mosquito-host-mosquito transmission chain cannot be maintained throughout the year. The primary suspects in this search have been snakes and bats since both of these animals exist with relatively low ambient body temperatures during a considerable portion of their life cycle. The investigations carried out with snakes and bats and reported in this thesis include: (1) the determination of the number of plaque forming units (PFU) of virus required in snake blood to infect a mosquito, (2) the changes in virus concentration in the blood of artificially infected snakes kept at room temperature for extended periods of time, (3) the number of infected mosquitoes required to infect a snake such that the snake can re-infect other mosquitoes, and (4) the ability of certain species of bats to harbor virus in their blood

and other tissues for extended periods of time, both at room and hibernating temperatures. In addition to the foregoing, this report will also describe the methods used in testing for virus the many animals that were collected in the field and also the results of these tests.

LITERATURE REVIEW

By 1930 malaria and yellow fever had been virtually eliminated from the North American continent. Epidemiologists and entomologists, therefore, became interested in the arthropod, in this case, mosquito-borne encephalitides, of which three important types are known to exist in North America: St. Louis encephalitis (SLE), Eastern equine encephalitis (EEE) and Western equine encephalitis (WEE).

Although much has been written in the literature concerning all three of these viruses, this review will be concerned primarily with the more commonly known publications treating the epidemiological aspects of the disease Western equine encephalitis; but when relevant, some of the other arthropod-borne viruses will be discussed.

I. HISTORY

Known epidemics of encephalitis in horses have occurred in the United States since 1831 (Hanson, 1957), however, it was not until 1930 that the etiological agent was isolated from the brain of a horse by Karl F. Meyer (1931). After isolating the virus Meyer (1932) made known the possibility of human involvement following WEE infection in horses, and subsequently Howitt (1938, 1939) was able to isolate the same virus from the brain of a child and from human blood, thus proving the importance of this virus in causing diseases among human as well as zootic populations. Of possible diagnostic importance is the report by Gwatkin (1943) on the isolation of WEE virus from the spinal fluid of an infected human.

II. EPIDEMIOLOGY

A. Epidemics

Serious epidemics of equine encephalitis have occurred among animals in the United States since 1847, probably involving both EEE and WEE viruses. In 1912, 35,000 horses and mules were lost in Kansas and Nebraska alone in an epidemic involving 17 states (Meyer, 1933a). Over a 16 year period, 1935-51, a total of 33,563 equine deaths were reported (Simms, 1951). The number of reported cases during the peak years of this period were as follows: 1935, 23,512; 1937, 173,889; 1938, 184,662; 1940, 16,941; 1941, 36,872 and 1944, 15,590. The incidence of this disease in horses has gradually dropped since 1944, probably because of a drop in overall horse population and in more recent years the use of an effective vaccine.

In 1941 the most severe human epidemic involving WEE virus occurred in North Dakota, Minnesota and adjacent Canadian provinces, where more than 3,000 people were infected of which approximately ten per cent were reported to have succumbed (Leake, 1941). In the early 1950's a major outbreak of human encephalitis occurred in California and in 1958 approximately 60 cases were reported in Utah, six of which ended in death (Gebhardt and Hill, 1960). More recently, one death in 1962 was attributed to WEE infection in the Salt Lake area (Jenkins, 1963).

B. Geographic Distribution

Western equine encephalitis occurs mainly in the United States and Canada west of the Mississippi River and in Illinois and Wisconsin. Sporadic virus isolations have been made in Rhode Island and Florida (Holden, 1955; Kissling, 1955; Alexander and Murray, 1957). The primary focus of infection seems to have changed from the Midwest during the 1930's to California in the 1950's. An endemic focus exists now over most of the Western United States and in particular along the Pacific Coast (Hammon, 1945a; Ferguson, 1954). The area of distribution corresponds to the range of the culicine mosquito Culex tarsalis except where sporadic isolations have been made in the Eastern part of the United States (Hess, 1958). The scope of infection by WEE virus was widened considerably when Meyer (1935) and Howitt (1935) reported Argentine equine encephalitis was identical to WEE virus.

C. Seasonal Occurrence

For the most part, infections with WEE virus occur during the hot part of the summer with some overlap into the fall. To show this a rating scale has been drawn up by the Bureau of Animal Industry. This group has selected three seasonal periods; pre-epizootic, epizootic and post-epizootic. Simms (1951), using this rating scale, reported the number of equine cases per month as follows: Pre-epizootic - January (7), February (6), March (3), April (11), and May (41); epizootic - June (44), July (153), August (218), September (303) and October (141);

postepizootic - November (39) and December (7). It is safe to assume that the cases reported during the winter were from more southerly states. In summary, one can say the disease prevails chiefly from early June to mid-September in the Western United States. In the Salt Lake area the disease is usually seen in horses and man in July and August.

In the San Joaquin Valley epidemic in 1931, the number of cases rose considerably when the temperature ranged between 90° and 100° F (Meyer, 1933b). In Kansas and in recent outbreaks in Canada high humidity had been recorded (Ferguson, 1954). It is now known that temperatures 80° F or above along with high humidity are important factors in the survival of the mosquito C. tarsalis and in the maturation of the virus in these mosquitoes (Hess, 1958).

D. Host Range

In addition to horses and humans, which are considered by most investigators to be incidental hosts for WEE virus, this virus has been found to occur naturally in the ground squirrel (Gwatkin, 1940 and Lennette, 1956) prairie chickens, deer (Cox, 1941) and pigs (McNutt, 1943). Hess and Holden (1957) reported that WEE virus has been isolated from approximately 20 species of birds and six species of mammals and that WEE antibodies have been detected in more than 75 species of wild birds and a half dozen species of wild mammals in addition to most of the common domestic birds and mammals. The actual determination of which species of animal is important in maintaining WEE virus in

nature has been somewhat complicated. However, with the suggestion by Ten Broeck (1935 and 1938), that birds might be the natural host for these viruses, a subsequent series of isolations were made from these animals in nature.

The first isolations involving an arthropod-borne virus, in this instance EEE virus, were made by Tyzzer (1938) from sick ring-necked pheasants and by Fothergill and Dingle (1938) from a pigeon.

Following the reports that the Eastern researchers were able to isolate EEE virus from birds, Hammon (1941a) conducted an antibody study on the sera of small birds and animals collected during the Yakima Valley epidemic in Washington. Antibody to both SLE virus and WEE virus was found in several species of wild birds including horned owls, sparrow hawks and quail. Several killdeers showed prior infection to WEE whereas mourning doves were immune to SLE. Among the small animals from which sera were collected, one of eight meadow mice, Microtus montanus, one of four deer mice, Peromyscus maniculatus, and two of three weasels, Mustela frenata were positive for WEE antibody. Although these studies gave evidence for both mammals and birds being previously infected by WEE and/or SLE virus, the birds seemed the more likely source of virus for infected mosquitoes during the epidemic.

WEE virus was first isolated from a bird, Tympanuchus cupido americanus (prairie chicken), by Cox (1941). The bird was shot in August on a farm near Rugby, North Dakota, where an extensive

encephalitis epidemic was occurring in horses and man. Since then Reeves (1944 and 1955a), Hammon (1945a), Eklund (1954), Holden (1955) and Kissling (1957b), among others have proven birds to be the important summertime endemic and epidemic reservoir for WEE as well as the other arbo-encephalitis viruses in nature. Reeves and Hammon (1944) found large numbers of C. tarsalis mosquitoes in domestic chicken houses in the Yakima Valley, Washington, thus implicating domestic chickens as the primary source of virus for mosquitoes; however, precipitin tests (Reeves and Hammon, 1944) and field biting host preference tests (Dow, et al., 1957) indicate that in addition to birds, other hosts such as a variety of mammals and even reptiles might possibly serve as virus reservoirs for infecting mosquitoes. After experimentally infecting chickens (Hammon, 1946) and wild birds (Hammon, 1951), this author suggested that wild birds rather than domestic fowl would more likely serve as a source for infectious virus--sparrows, finches and blackbirds being the most important. In some individual wild birds, virus was recovered from blood as late as nine and ten days after inoculation; however, most recoveries were made during the first four days. Maximum titers occurred during the first two days. Domestic fowl were shown to circulate sufficient virus in their bloods to infect mosquitoes, but the virus titers and the duration of viremia were less than those obtained in wild birds. Dow (1957) gave further insight into the importance of wild bird reservoirs when his field tests indicated

that even though mosquitoes were attracted in larger numbers to domestic fowl, the percentage of those taking blood meals was considerably higher among those attracted to wild birds. His explanation was that the larger birds give off more carbon dioxide and this suffices to attract more mosquitoes; the ability of the mosquitoes to feed on the larger domestic birds, however, is somewhat less than their ability to feed on the smaller wild ones. In fact, unpublished observations by Blackmore and Dow showed that feeding rates of C. tarsalis on the small inactive nestlings were much higher than on adult birds (Hess, 1957). Sooter (1951) was able to isolate WEE virus from nestling redwinged blackbirds and magpies captured near Nunn and Greeley, Colorado. Since this time many isolations have been made from wild birds in nature, thus establishing them as the most important vertebrate hosts of WEE virus in nature (Johnson, 1960).

E. Vectors and Viral Transmission of WEE

The first notable discovery concerning the transmission of WEE virus was made by Kelser (1933) who was able to demonstrate the first laboratory transmission of equine encephalitis. Working with horses, guinea pigs and the mosquito Aedes aegypti, of Phillipine Island origin, he was able to transmit the disease from infected guinea pigs to normal guinea pigs, from infected guinea pigs to a horse and from the horse back to guinea pigs. The mosquitoes transmitted virus as early as five days after taking a blood meal and probably remained infective the rest of their lives.

In similar experiments the virus of WEE was also found to be capable of being transmitted by the mosquito Aedes vexans (Kelser, 1935), a tick Dermacentor andersoni (Syverton, 1937) and the mosquito Aedes taeniorhynchus (Kelser, 1937). Syverton (1941) was also able to demonstrate transovarian, or hereditary, transmission of WEE virus in the laboratory by ticks (Dermacentor andersoni). Although successful transmissions of the virus by mosquitoes and other arthropods was accomplished during the 1930's and early 1940's, none of these species of animals was considered to be important natural vectors because of the lack of field evidence.

In 1940 the first isolation of WEE virus from an arthropod in nature was made in Kansas from the body of a cone nose bug, Triatoma sanguisuga (Kitselman, et al., 1940). In the following year WEE and/or SLE viruses were isolated from the mosquitoes Aedes dorsalis (Hammon, 1945b)and Culex tarsalis (Hammon, 1941b) in California; in 1942, WEE virus was isolated in the state of Washington from the mosquitoes Culex pipiens, C. tarsalis, Culiseta inornata and Anopheles freeborni (Hammon, et al., 1945c); in Canada in 1944 WEE virus was isolated from Culex restuans (Norris, 1946). In addition to these, WEE virus has been isolated from the mosquitoes Culiseta melanura and Mansonia perturbans, however, most of the isolations made from mosquitoes were rare except those made from C. tarsalis. In 1942 Hammon et al. (1942) tested, by animal inoculation, 15,610 arthropods from the Yakima

Valley, Washington. Frequent isolations of Western and St. Louis viruses were made only from C. tarsalis mosquitoes during the months of July and August. Hammon (1943a), tested experimentally the capabilities of C. tarsalis as a vector of WEE in the laboratory and was able to show transmission of WEE infection from a guinea pig and duck to chickens after incubating previously engorged mosquitoes for ten days. The mosquitoes remained infective for at least 30 days. Prior to this Trager (1938) and later Chamberlain and Sudia (1957a) were able to grow virus on mosquito tissue grown in cell culture and were able to show an increase in titer of the virus grown on such tissues. Chamberlain (1957a) working in his laboratory has also shown the mosquito C. tarsalis to be an efficient vector of WEE virus. He reports when C. tarsalis were given a blood meal nearly all the mosquitoes became infected and 85 per cent of these were capable of transmission. Blood meals with a virus titer of 10 mouse LD_{50}'s would infect from one to five per cent of the mosquitoes ingesting it, 10,000 times as much virus could be recovered as had been ingested. Previous to 1944 no direct association between the most probable vector, C. tarsalis, and its likely source of virus had been made; however, Hammon et al. (1943b) after reviewing the evidence up to that time, hypothesized that the host animal serving as a reservoir of virus for mosquitoes must fulfill these requirements: (a) they should be abundant, (b) they should show no apparent signs of infection, because no epizootics had ever been observed except in horses and

horses were relatively few in number, (c) they should have as a result of small peripheral inoculation, a reasonably large amount of virus circulating in the blood for more than a fleeting period of time, (d) they could theoretically be birds, because in an area where epidemics occur annually, the reservoir animal should be one which does not bestow a first season's protection to its offspring by maternal transmission of antibody as frequently occurs in mammals. The following year Reeves and Hammon (1944), as mentioned previously, noticed the presence of large numbers of C. tarsalis mosquitoes in chicken coops located in the Yakima Valley, Washington. The conclusion was then drawn that domestic fowl must be the preferred host for C. tarsalis. These findings coupled with frequent isolations of WEE virus in nature from mosquitoes indicated that C. tarsalis was the primary carrier of infection. In summary, the proofs for C. tarsalis serving as an excellent vector for both endemic and epidemic transmission of WEE are: (1) the mosquito is widely distributed and abundant, (2) freely feeds on man, horses and birds, (3) is very susceptible to infection and (4) transmits WEE virus with a high efficiency.

Chamberlain (1958) summarized the virus-vector-host relationship to man, thusly: "When an exceptionally high population of a susceptible mosquito occurs, associated with a general low level of immunity in the bird population, feeding specificity becomes a less important factor.

The vertebrate host range is now extended, and man and horses may be included in it."

In addition to mosquitoes, ticks and cone nose bugs, mites were also considered as possibly being of some importance in the natural transmission of WEE. Reeves (1947) and Miles (1951) recovered WEE virus from wild bird mites, Liponyssus sylviarum and Dermanyssus americanus respectively. Following field and laboratory studies by Reeves (1955b) Chamberlain and Sikes (1955), Sulkin (1955) and Chamberlain (1957b) the possibility of mites serving as an important vector in nature was dropped, primarily because of the fact that measurable virus persisted for less than two days in these animals and all attempts to transmit virus after this period failed.

In summary, C. tarsalis remains the principal vector of St. Louis and Western equine encephalitis viruses in the Western part of the United States. The appearance of Western equine infections in the Eastern part of the United States suggests that another vector may be important in these areas.

F. Overwintering

Wild birds and mosquitoes have been shown to be the principal animals in propagating Western equine virus in localities where this disease is endemic during the summertime.

The problem remaining is one of determining the whereabouts of WEE virus during the winter months, especially in cold areas when

transmission of the virus is impossible because of vector hibernation. Attempts by Kissling et al.(1957a) to isolate virus from wild birds during the winter and spring failed. This group tested a total of 1,503 migrating birds in the South Eastern United States and found no virus. The incidence of antibody in the migrating birds was lower than in native birds. In laboratory studies Reeves et al.(1958a) were able to demonstrate chronic latent infections in wild birds with WEE virus. Two-hundred-eighty-four (284) birds were inoculated with virus, after which their tissues were tested periodically. Virus was isolated from eight of the birds from one to ten months after infection. Although this study indicates the possibility that latent infections in birds might provide a winter reservoir, there has been no corroboration of their findings in the field.

Mosquitoes have also been considered by many workers to be the possible overwintering reservoir of WEE virus in nature. Blackmore (1956) has reported the only isolation of WEE virus from a pool of hibernating C. tarsalis mosquitoes taken from a cave in Colorado. Only one of the fifty pools collected during December, January and February was positive. In California where the climate is usually more mild than it is in the northern endemic area of WEE infection, Reeves et al. (1958) were not able to isolate virus from hibernating mosquitoes from the middle of November to the middle of January, even though large samples were taken during these months. It is interesting to note from their

studies that the virulence of the virus seems to have been somewhat attenuated compared to isolations made during the warmer part of the year. For example, two of three January isolations and one of three February isolations would not kill adult mice by intracranial inoculation until from three to six passages in embryonated eggs had been made. The antibody response of viruses isolated in the winter was lower in chicks than the response elicited by those isolated during the summer. The possibility of avirulent strains of virus constituting a winter reservoir now arises, which would complicate the search for such a reservoir. In conjunction with this thought, Dunayevich and Johnson (1961) have reported the isolation of a relatively avirulent Western equine virus strain from a bird in California. The virus became less virulent on successive cloning and also, unfortunately, lost most of its immunogenic properties.

Under experimental conditions Barnett (1956) was able to keep infected C. tarsalis alive at 44° F for 43 days in a root cellar; later Bellamy et al. (1958) showed that WEE virus could persist in C. tarsalis for 41 days at 55° F after incubating the mosquitoes to the infective stage at 75° to 84° F. In the same study these authors showed that WEE virus was able to survive 113 days in C. tarsalis incubated during the winter in an unheated cellar. C. tarsalis also completed extrinsic incubation and transmitted WEE virus to chickens after being incubated at winter temperatures for 97 and 109 days. The authors concluded that C. tarsalis could carry WEE virus through the winter by serial transmission.

At the same time Bellamy was carrying out his study, Rush (1958) was gathering information from a natural hibernation site of C. tarsalis in the Columbia River Basin, Washington, that indicated blood-fed females of this species were seldom able to survive winter hibernation. Since this observation other workers have found while attempting to hibernate mosquitoes, especially at low temperatures, that blood-fed females do not survive (Bellamy et al., 1958; Bennington et al., 1958). The inability of blood-fed C. tarsalis mosquitoes to survive, especially in colder climates, has all but ruled out this species as a possible winter reservoir.

The possibility of certain mammals serving as a winter reservoir for WEE virus has been considered; however, with the exception of bats and squirrels, very little work has been done along these lines. Kissling (1958) states, in summary, that mammals do not circulate enough virus in their bloods to be an important source of virus for mosquitoes.

Bats have been suspected as carriers of certain virus diseases for many years. Reports by Pawan (1936), Verteuil and Urich (1936), Gilyard (1945), Johnson (1948), among others, have quite clearly indicated that vampire bats have been sources of rabies virus infection to cattle and other domesticated animals as well as to man. Rabies virus has also been isolated from other than hemophagus bats through the years. Haupt and Rehaag (1921) found that "leaf nosed" fruit eating bats in Brazil were infected with rabies. In the United States, Venters et al.

(1954) were the first to diagnose rabies in a Florida yellow bat (Dasypterus floridanus), since then rabies virus has been isolated from the Mexican free-tailed bat, Taderida mexicana, (Enright, 1955) and the large brown bat, Eptesicus fuscus pallidus, (Public Health Service Rocky Mountain Laboratories). Hurst and Powan (1936) believe that there are six forms of rabies seen in bats: (1) furious form followed by paralysis and death, (2) paralysis, not preceeded by a furious form, with death, (3) furious form with recovery, (4) furious form followed immediately by death, (5) sudden death with no symptoms and (6) symptom free carrier. The last of these states, the symptomless carrier state, has led other investigators to study the ability of these animals to carry other viruses latently.

Corristan et al. (1956) were able to infect bats with Venezuelan equine encephalitis virus. The bats harbored the virus for 90 days at hibernating temperatures without ill effects. Bats incubated at room temperature were found to have a viremia for 26 days, the maximum titer of which was 10^6 mouse intraperitoneal LD_{50}/ml. The titers at 10° C ranged from 10^2 to 10^3 MIP LD_{50}/ml.

LaMotte (1958) infected several species of bats with Japanese B encephalitis virus. The average duration of the viremia in these bats at room temperature was from three to nine days with an occasional bat showing a viremia up to fifteen days. LaMotte was unable to demonstrate virus in the brains of infected bats even though their blood was

highly infective. He was able to infect one bat orally by feeding it three previously infected mosquitoes. All other attempts to infect by this route failed. Successful transmission of the virus from mosquitoes to bats and vice versa was accomplished. When previously infected mosquitoes were put in with bats hibernating at 10° C, the mosquitoes were able to transmit the virus to bats. Hibernating bats harbored the virus for 107 days and when the animals were returned to room temperatures, the viremia rose to a sufficient magnitude to infect mosquitoes placed with them for feeding.

The isolation of a virus antigenically related to St. Louis encephalitis was reported by Burns and Farinacci (1956). This virus was recovered from bat salivary gland tissue by inoculating baby mice. The virus was not neutralized by rabies antiserum; however, specific SLE quinea pig antiserum consistently fixed complement with this virus. The bat antisera would not fix complement with a known SLE virus strain. Other arbovirus antisera would not react.

The evidence presented for bats seems to indicate that these animals have certain unknown mechanisms operating within themselves that enable them to carry latent virus infections for considerable periods of time. The isolation of a virus similar to another arthropod-borne virus adds credence to the belief that these animals should be investigated further as a possible overwintering reservoir of WEE virus.

In addition to bats, certain reptiles have also been considered as a possible reservoir for WEE in nature. Rosenbusch (1942) inoculated snakes of several species common to Argentina and found some to harbor the virus for at least 39 days. Virus was recovered from the brains of all snakes that became infected, virus was not recovered from the bloods of these snakes at the times tested. In the same study toads did not reveal the presence of virus after seven days.

More recently Thomas et al. (1958) showed infected garter snakes (Thamnophis spp.) to be capable of carrying WEE virus for 36 days. Gebhardt and Hill (1960) have demonstrated the ability of this same genus of snakes to harbor WEE virus under hibernating conditions for at least 139 days, with unpublished data showing WEE virus being present for 160 days. During the hibernating period the viremia of these animals declined steadily; however, after the snakes came out of hibernation the blood titers increased. In another report, Thomas et al. (1962) were able to infect snakes with WEE virus by the mosquito C. tarsalis in the fall, hibernate the snakes through the winter, and then reinfect other animals in the spring from these same snakes using fresh C. tarsalis. If the snakes were kept at room temperature, virus was isolated one week after emergence; but if snakes were kept at their natural hibernating site, virus was not isolated until five weeks after emergence. A viremia in some of these snakes could be detected up to ten weeks after emerging.

The ability of viremic snakes to infect mosquitoes in the spring after natural hibernation increases the significance of the hypothesis that these animals might be important in the yearly maintenance of WEE virus in cold climates.

MATERIALS AND METHODS

I. SOLUTIONS AND MEDIA

A. Chick Embryo Cell Growth Medium (Complete)

The growth medium used for cultivating chick embryo cells or preserving and diluting blood and for preparing brain homogenates was composed of Earles' balanced salt solution, 0.4 per cent lact-albumin hydrolysate, 5 per cent calf serum, 0.5 per cent of a 4.5 per cent $NaHCO_3$ solution, 100 units per ml penicillin and 100 ug per ml streptomycin. The medium was sterilized by Seitz filtration using positive pressure.

Earles' balanced salt solution was made up in 10 X concentrations and stored unsterile at 4° C until used. Its formula is:

Phenol red	0.02 g/liter
NaCl	6.8 g/liter
KCl	0.4 g/liter
$NaH_2PO_4 . H_2O$	0.14 g/liter
$MgSO_4 . 7H_2O$	0.2 g/liter
Anhydrous $CaCl_2$	0.2 g/liter
Glucose	1.0 g/liter

B. Phosphate Buffered Saline (PBS)

Phosphate buffered saline, pH 7.2, plus ten per cent heated normal rabbit serum was used as diluent in some instances for making up ten per cent blood or brain suspensions for storage at -30° C. PBS minus

Ca++ and Mg++ (PD) was used in preparation of the chick embryo cells. The PBS was prepared according to Dulbecco and Vogt (1954) as follows:

NaCl	8.0 g/liter
KCl	0.2 g/liter
Anhydrous Na_2HPO_4	0.15 g/liter
Anhydrous KH_2PO_4	0.2 g/liter
$MgCl_2 \cdot 6H_2O$	0.1 g/liter
Anhydrous $CaCl_2$	0.1 g/liter

The $CaCl_2$ was added last with much mixing and if 10X PHS was desired, the MgCl and CaCl was omitted until just before use. Both the PD and PBS were made up in 10X concentrations and stored at room temperature for short periods of time after Seitz filtration.

C. Trypsin Solution 0.25 Per Cent (Youngner, 1954)

Two and one-half grams (2.5) of a 1:300 trypsin preparation (Nutritional Biochemical Corporation) was dissolved in 1000 ml of PD and the solution sterilized by Seitz filtration. The resulting solution was stored in the deep freeze until used, at which time it was warmed in a 37° C water bath.

D. Neutral Red Solution

Neutral red (1:5000) was dissolved in 0.85 per cent NaCl and sterilized in the autoclave.

E. Purified Agar

Agar for use in the overlay medium was purified by repeated washings with distilled water, acetone and ether. A similar method was described by Dulbecco and Vogt (1954).

F. Agar Overlay Medium

A 1.8 per cent agar solution was made up using purified agar and distilled water. After sterilizing in the autoclave the medium was allowed to equilibrate at 42.5° C in a temperature controlled water bath. The agar overlay medium was prepared just before use by mixing equal volumes of the 1.8 per cent agar solution and sterile 2X concentrated Earle's complete growth media containing six per cent calf serum.

G. Nutrient Agar and Blood Agar

Nutrient and blood agar plates were used in checking bacterial infection and/or contamination of blood or other tissue samples. The formulae for these media were as follows:

1. Nutrient Agar

Peptone	5 g
Beef extract	3 g
Agar	15 g
Distilled water	1000 ml

2. Blood Agar

Bacto tryptose	20 g
Bacto dextrose	2 g
NaCl	5 g
Disodium phosphate	2.5 g
Agar	20 g
Human blood (citrated)	20 ml
Distilled water	1000 ml

II. VIRUS SOURCE

The original WEE virus (Ushijima, 1962) used in these studies was isolated from human tissues by inoculation into one-half-day old "wet" chicks. Subsequently, the virus was passed several times through mice by intracerebral inoculation prior to infecting garter snake embryo cell cultures maintained at 25° C. These cells developed extensive cytopathic changes. Fluid from the infected snake embryo cell cultures were then used as a source of virus to infect primary chick embryo cells (CEC). After the tenth passage a plaque measuring 5 mm in diameter and labeled W-4 was selected and the virus progeny isolated, recloned, and used for further investigations. After several more passages through CEC monolayers, two variants were isolated and designated as SP-6 and LP-7 viruses. The SP-6 produced plaques averaging 2 mm in diameter while the average diameter of the LP-7 plaque was 8 mm. The SP-6 virus plaques were irregular around the

margins while the LP-7 plaques were smooth and "entire." Both strains are antigenically homogeneous with regard to neutralization by specific antibody.

III. CHICK SOURCE FOR VIRUS ISOLATION

The baby chicks used in these studies were acquired from a local hatchery. These chicks were all pure bred male cockerels of the Hi-Line strain and they were used "wet" (i. e. less than one day old) at which time they were highly susceptible to WEE virus (Hill et al., 1963). Inoculated chicks were not given food nor water for three days.

IV. MOUSE SOURCE FOR VIRUS ISOLATION

The suckling 6-8 day old mice used in these studies were obtained from a colony of white Swiss CFW mice bred in the animal quarters, Department of Microbiology, University of Utah, College of Medicine. After the mice were injected they were marked and placed into small galvanized tins, two mothers and 16 sucklings per tin. The mice were fed Purina Laboratory Chow plus ample quantities of wheat and water; their shavings bedding was changed every five days.

V. BAT SOURCE AND CARE

Five species of bats*, Pepistrellus hesperus "Western pepistrel," Myotus lucifugus "little brown bat," Myotis velifer "cave myotis,"

* Bats were kindly identified by Dr. J. W Twente, Dept. Zoology, University of Utah.

Plecotus townsendii "lump nose bat," and Taderida mexicana, the "Mexican freetail bat" were captured at various times and in different places in Utah during the summer and fall of 1962.

All bats were kept in cylindrical cages 12 x 18 inches constructed of bronze screen. The bottoms were left open so that feeding and watering could be accomplished by lifting the cage off its base. From one to fifteen bats were kept in individual cages. All bats were fed water and a diet containing one part meal worms, one part cottage cheese, one part banana, one part hard-boiled egg yolk and a few drops of vitamins (A, B, C D, and E), wheat germ, oil and iron. As will be noted later, all bats except the lump nose bat, P. townsendii, survived on this diet, the lump nose bats lost weight gradually and eventually died.

Bats were hibernated in a special refrigerator maintained at 6° C. A fan gently blew fresh air into the top part of the hibernating chamber by means of a rubber tube placed through a hole bored in the wall of the refrigerator; exhaust air was removed through another rubber tube similarly constructed near the bottom of the chamber. Cylindrical wooden cages covered with bronze screen housed the bats within the refrigerator. Fresh water was given to the bats periodically, but food was not offered.

VI. SNAKE SOURCE AND CARE

Garter snakes caught and tested were of two species, Thamnophis o. vagrans and T. n. sirtalis parietalis, as identified by Dr. A. M. Woodbury, Department of Zoology, University of Utah. These snakes were collected from marshy areas bordering the Great Salt Lake between Salt Lake City and Ogden, Utah. Horse and human encephalitis infections have been reported from this area in recent years. Some of these snakes have been maintained for approximately two years in screened galvanized tanks on a diet of live mice fed to them about every six weeks.

VII. SNAKE BLEEDING

After anaesthetizing a snake for seven or eight minutes with diethyl ether, the snake was washed with running tap water and placed on a dissecting board with the ventral surface up. If the snake were properly anaesthetized, it would remain this way for the period of time necessary for bleeding. The area over the heart, located approximately one-fourth of the distance from the snout to the anus, was then disinfected with 70 per cent alcohol. Next, the exact location of the heart was found by noticing the slight pulsating movements of the abdomen in this area. These pulsations were more evident if light were allowed to reflect off the surface in this area. For best bleeding results, however, the apex of the heart had to be located. This structure was most visible under the aforementioned conditions when the

animal exhales. After the apex was located, this region was grasped with the fingers and thumb of the left hand and while feeling the heart beat a five ml syringe fitted with a 22 gauge needle was inserted with the right hand between the scales about 1 cm caudal to the apex. The needle was then worked forward almost parallel to the body toward the apex located between the fingers. A slight suction was held on the syringe so that the blood would well up into the barrel when the ventricle was punctured. Two to three mls of blood could be taken without serious harm to the snake. The blood collected was placed in a small centrifuge tube and allowed to partially coagulate which usually took about three to five minutes. When the blood reached the proper consistency as determined by experience it was centrifuged in a clinical centrifuge at approximately 3500 RPM for ten minutes. The liquid serum was removed and placed in sterile vials; when small amounts of whole blood were taken for virus isolations, 1:10 dilutions were made in complete growth medium. Sterile filter paper disks were also soaked with blood at the time of the bleeding for use in antibody studies.

VIII. BAT BLEEDING

All bats were bled by cardiac puncture using a two ml syringe fitted with a 25 gauge needle. The needle was inserted through the diaphragm up under the ribs and from 0.1 to 0.2 ml of blood was taken without injury. The blood specimens were diluted with complete growth

medium as previously described. Sterile filter paper disks soaked with blood were also prepared for antibody studies.

IX. BRAIN PASSAGES

Part of the virus isolation and identification procedures involved the passing of brain tissue from sick previously injected animals to normal baby mice or "wet" chicks. This technique was used both as a spot check for the presence of virus in certain of the studies involving animals and also in isolation attempts. The procedure was carried out thusly:

Previously injected moribund animals were labeled and placed in the deep freeze until brain passages could be made. After brief storage the brains were removed aseptically, triturated, and suspended in complete growth medium to a final concentration of approximately ten per cent. This suspension was inoculated intracranially into either "wet" chicks or "suckling" 6-8 day old mice. A part of the brain was also streaked on blood agar and nutrient agar plates for bacterial analysis prior to being triturated in growth media. When required, several brain passages were made.

X. MOSQUITO REARING

The C. tarsalis mosquitoes used in these experiments were obtained from a colony which has been maintained at the University of Utah since

1961. The original stock was obtained from the Encephalitis Section, U. S. Public Health Service, Greeley, Colorado.

These mosquitoes were reared in a fairly air-tight, moisture-proof room with tables and shelves present for supporting larvae pans and infecting cages. The room also contained a bronze wire screened adult breeding cage, 2 x 2 x 4 feet, which had two hand entry holes protected by gauze sleeves for entering into and manipulating within the cage.

Accessories within the cage consisted of a small box with a slit down the front and several types of plants having heavy foilage. Both the box and the plants provided the mosquitoes with dark natural hiding places during the day.

White porcelainized pans, 7 x 11 x 2 inches, were used to raise the larvae outside the adult breeding cage. According to the size of the adult colony and preplanned withdrawals of adults or pupae for use in experiments, a cycle was maintained in which the number of pans set up each day with egg rafts (usually two to five per pan) provided the necessary numbers of adults for both colony maintenance and experimentation. When studies were to be run, as many as six pans per day were set up for extended periods of time. Distilled water was used as the rearing medium.

All larval pans and other equipment in the rearing room was kept clean and steam cleaned without the use of soap or detergents. Lysol

and acetonewere used for cleaning cages, benches, etc., but not larvae pans. Distilled water was used as a rinse.

Relative humidity was kept at 70 per cent by means of an evaporative air contitioner regulated by a humidostat obtained from Minneapolis Honeywell, Minneapolis, Minnesota.

The temperatures in the insectory were maintained at 80° F by use of electric thermostatically controlled heaters.

Overhead fluorescent and regular lights were kept on for a minimum of 13 1/2 hours a day. An automatic switch turned the lights on at 2:00 a. m. and off at 4:00 p. m.

The adult mosquitoes were fed using gauze pads saturated with ten per cent Karo syrup dilutions or by means of whole raisins soaked overnight in distilled water. The larvae were fed Purina mouse chow and Twill*, a concentrated protein dietary product. The Twill was fed in increasing amounts after the eighth day.

Scum resulting from bacterial growth in the larval rearing water was removed daily by dipping with small wire screens. When tetracyclines were experimentally used to reduce bacterial growth, there was significant increase in the total number of pupae produced per pan (St. Jeor, 1963).

In breeding the mosquitoes, chicks one to seven days old, for use as a blood source, were placed inside the breeding cage in a tight wire enclosure. Two or three days after the mosquitoes had taken a blood

* Nutritional Service, Inc., P. O. Box 92, Cedar Rapids, Iowa.

meal, the egg rafts were removed and placed either in larvae pans or a 4° C refrigerator. Eggs remained viable in the refrigerator for up to eight days. When the egg rafts were placed in the larvae pans, not more than 3/4 of an inch of distilled water was present. As the larvae matured, the depth of the water was increased up to the top of the pan. Two egg rafts usually provided from 400 to 600 larvae of which approximately 70 per cent survived to become adults. The aquatic stage was completed in 12 to 15 days.

Mosquito infecting experiments were conducted in Plexiglass framed 8 x 12 inch square cages covered with Lumite Saran Screen. * On one side of the cages an eight inch diameter hand entry hole was made to which a protective gauze sleeve was attached. Direct lighting by means of desk lamps was kept on the pupae placed into these cages until they emerged into adults.

In the transmission studies all male mosquitoes and females not taking blood meals were removed from the cages. The animals to be exposed to mosquitoes were placed into the cages just before dusk and removed the next morning. Blood samples were taken prior to the first exposure and immediately after all other exposures. The infected mosquitoes were maintained on liquid sugar solution and raisins during the incubation period. Temperature and relative humidity was kept constant at 80° F and 70 per cent.

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* Chicopee Manufacturing Corporation, Buford, Georgia.

XI. CELL CULTURE TECHNIQUES

A. Preparation of Chick Embryo Cell Cultures

The cell cultures used in these studies were prepared in the following way:

Ten day old chick embryos were removed aseptically and placed into a petri dish containing PD solution. After severing the embryos' heads, wings and feet with a scissors the remaining bodies were transferredto a dry dish where they were minced with a scalpel before being transferred to a trypsinizing flask. After washing three times with PD, trypsinization was started with the addition of enough fresh trypsin to cover the tissues. The flask was then placed on a magnetic stirrer for approximately 5 minutes, and after allowing the cells to settle, the supernate was poured through a sterile gauze covered funnel into a flask previously placed into an ice bath. Immediately after the first supernatent was decanted approximately 20 or more mls of inactivated calf serum was added to the filtered cells to inactivate the trypsin. Trypsinization of the minced tissue was repeated four times and the resulting cell suspensions pooled. The cells were harvested by centrifugation in 250 ml bottles after which they were resuspended in a known volume of growth medium. The cells were counted with a hemocytometer, the desired dilution made and three or five mls of the cell suspension containing approximately 2.0×10^6 cells per ml inoculated either into screw-capped 16 x 125 mm tissue culture tubes or 60 mm pyrex glass

or plastic petri dishes (Falcon Plastics, Los Angeles, California). After incubating the cultures for 48 hours at 37° C in an atmosphere of approximately 5 per cent CO_2 in air, they were checked for monolayer confluency and inoculated with virus suspensions.

B. Plaque Assay Technique

Quantitative determination of the number of virus particles in certain blood samples were carried out using the plaque assay technique. In this technique two-tenths ml of the appropriate virus dilutions was added to established plate cultures, adsorption was carried out at 37° C for one hour in the presence of five per cent CO_2 after which five ml of the agar overlay medium were added to each plate. After 24 hours of incubation under the previously mentioned conditions, 2.0 ml of a 1:5000 neutral red solution was added to the agar surface of the plates and allowed to diffuse through the medium for one hour. The excess neutral red was then removed and the plates were returned to the incubator. The plaques were counted six to 24 hours later and appeared as clear areas in a red background.

XII. VIRUS ISOLATION

Three methods for virus isolation were used in these studies. They were: (1) inoculation of chick embryo cell monolayer or tube cultures, (2) intracranial inoculation of "suckling" 6 to 8 day old mice in doses of 0.025 ml, and (3) subcutaneous inoculation of less than 12 hour old "wet" chicks, with doses of from 0.2 to 1.0 ml.

The blood and serum samples were always diluted when they were to be injected into baby mice or chicks; however, undiluted bat and snake bloods were used with the plaque assay method.

XIII. ANTIBODY DETECTION AND VIRUS IDENTIFICATION

All assays for antibody and virus characterization was done by use of virus neutralization tests in suckling mice, wet chicks and/or chick embryo cell monolayers. Serum-virus (100 PFU) mixtures were incubated at 37° C for 1 1/2 hours and then placed at 4° C for several hours prior to inoculation. Dilutions were made with growth medium. When the micro-method on chick embryo cell monolayers was used, sera were diluted drop-wise and then mixed with an equal volume of WEE virus (20 to 25 PFU/drop). After incubation, one drop of the mixture was added to chick embryo cell monolayers. Positive horse serum having a 50 per cent plaque reduction titer of 10^3, using 100 PFU of virus was used as a control. Normal negative sera from rabbits and horses were also used in the tests.

RESULTS

I. EXPERIMENTAL TRANSMISSION OF WEE VIRUS FROM SNAKES TO *CULEX TARSALIS* MOSQUITOES

Although the successful transmission of Western equine encephalitis virus from snakes to birds, birds to snakes and snakes to snakes by means of mosquitoes has been reported from other laboratories (Thomas and Eklund, 1958), no quantitative measurements, as far as is known, have been made on the number of infective virus particles essential in snake blood to infect a mosquito taking a blood meal.

Therefore, the following experiment was designed in which artificially infected snakes provided varying quantities of blood virus, as determined by plaque assay methods, to groups of female *C. tarsalis* mosquitoes. After fourteen days for maturation of virus in mosquitoes "wet" chicks were exposed to the insects to prove the ability of the mosquitoes to infect and on the fifteenth day each individual mosquito was triturated and injected into "wet" chicks to determine whether or not they actually contained infective virus.

The snakes for this study were infected and assayed for virus as follows:

Four snakes (No. 32, 73, 111 and 37) selected from a group of normal snakes were each infected with 150,000 PFU of mouse brain LP-7 WEE virus, 50,000 sc, 50,000 ip and 50,000 iv, before being exposed to mosquitoes at varied times. Blood samples were drawn for

virus assay prior to injection and periodically thereafter on the mornings following exposure to mosquitoes. The blood samples obtained were diluted 1:10 with sterile PBS plus ten per cent heated rabbit serum and stored at -30° C until assayed on CEC monolayer cultures. The results of these virus assays are shown in Table 1, Column III. It is noteworthy that there was considerable variation in the magnitude and duration of the viremias exhibited by these reptiles under the conditions of the experiment.

The preparation of the mosquitoes and infecting cages to be used in this transmission study started four days prior to the exposure of the mosquitoes to infected snakes when approximately 65 freshly emerged pupae were washed and placed into 250 ml beakers one-half filled with distilled water. These pupae were placed into individual infecting cages prior to emergence. After emerging the adult mosquitoes were fed ten per cent Karo syrup and raisins until two days before taking a blood meal, at this time the food and beakers were removed from the cages. During the time the mature mosquitoes were emerging from the pupae the cages were exposed to direct artificial light, since higher emergence values were obtained in this manner.

Prior to placing the mosquitoes with infected snakes all the male C. tarsalis were removed from the cages. All snakes were exposed to the mosquitoes from dusk until the following morning when all the female mosquitoes that had not taken a blood meal were removed. The

lights were turned off at 4:00 p. m. and on at 4:00 a. m. by means of an automatic switch. An average temperature of 80° F and a relative humidity of 75 per cent were maintained in the infecting room at all times.

The data concerning per cent survival, number of females and number of males emerging in each cage and the percentage of mosquitoes feeding on snakes are given in Table 2.

Calculations from these data showed that 83 per cent of the females, placed into cages with infected snakes took a blood meal; of these, 71 per cent survived to the end of the experiment at 15 days.

After the mosquitoes had fed on the infected snakes, Karo syrup and raisin feedings were resumed and a beaker of distilled water was placed into the cage for egg laying.

Fourteen days later the mosquitoes in each cage were given a second blood meal on a "wet" chick. Except for two, chicks from all of these cages died, with symptoms suggestive of encephalitis indicating there were infective mosquitoes present in at least ten of the cages. On the fifteenth day after the mosquitoes had fed on the infected snakes, they were removed from the cages using a suction tube and immediately frozen at -30° C in a sterile glass vial until they could be assayed individually for infective virus.

When assayed the mosquitoes were transferred one at a time from the vial into sterile 5 ml centrifuge tubes containing a small

amount of ground glass. Each mosquito was immediately ground in the glass using a sterile wooden applicator stick, one drop of cold growth medium was added and the tissues ground again. The homogenate was frozen in an acetone dry ice bath for a few seconds, removed and ground again. The freeze-grinding procedure was repeated twice after dropwise additions of growth medium. The final volume of the homogenate was 0.4 ml thus giving approximately a $10^{-2.3}$ suspension, since the mean weight of these female mosquitoes was found to be approximately 2 mg. The 0.4 ml volume was centrifuged at 5,000 RPM in a clinical centrifuge for six minutes, the supernatant removed with a micro-pipette and 3 drops (0.1 ml) diluted in 0.9 ml of complete growth medium.

When all the mosquitoes in each cage had been ground individually, the resulting suspensions, $10^{-2.3}$ and $10^{-3.3}$, were injected into "wet" chicks. One-tenth ml of the $10^{-2.3}$ suspension was injected sc into each of 2 chicks, while 0.1 ml of the $10^{-3.3}$ suspension was injected into each of 4 "wet" chicks. Since the dilutions were somewhat high, the death of one chick showing symptoms of encephalitis was considered evidence of viral infection in the original mosquito. Thirty uninoculated control chicks all survived for the duration of the test.

Since the time between hatching and inoculating chicks approached 18 hours, twenty-four control chicks were inoculated last with known WEE virus suspensions mixed and triturated with normal mosquito

tissues. A few drops of the virus suspension were added to each mosquito suspension after which the same procedure previously described was carried out. One hundred per cent of the inoculated chicks died. Five chicks injected with ground normal mosquito homogenates in growth medium all survived.

The results of these studies are shown in Table 2 and Figure 1 where the percentage of mosquitoes infected as determined by injection of "wet" chick with a dilution of $10^{-2.3}$ or greater, is plotted against the titer of virus in the blood of snakes on which these mosquitoes fed. The infective dilution of $10^{-2.3}$ or greater was based upon the weight of the mosquito and the volume of fluid required to inject the chicks. The mid-infective dose (ID_{50}) for mosquitoes was found to be approximately $10^{5.2}$ PFU/ml of blood with 95 per cent confidence limits between doses of $10^{3.9}$ PFU/ml and $10^{6.2}$ PFU/ml. The weighted regression line Figure 1 as well as the 95 per cent confidence limits pertaining to this line were calculated using the probit transformation method Geigy (1956).

TABLE 1

The percentage of mosquitoes infected by various quantities of WEE virus present in snake blood.

Snake No. injected with 1.5×10^5 PFU of WEE virus.	Time in hours from injection of virus to time of feeding mosquitoes.	Blood titer of snake log PFU per ml.	Number of mosquitoes feeding by observing swollen abdomens.	Number of mosquitoes infected by a $10^{-2.3}$* or greater dilution.
32	65	7.83	28/30**	14/17***
	113	7.49	25/30	14/16
	161	6.78	27/30	11/19
	311	Neg.		
73	53	5.78	27/30	5/16
	101	4.85	19/30	7/15
	149	3.50	23/30	5/18
	290	Neg.		
111	41	2.48	27/30	5/16
	89	Neg.	28/30	0/26
	137	Neg.	15/30	0/11
	280	Neg.		
37	29	3.48	28/30	4/17
	77	7.43	24/30	15/19
	125	7.04	26/30	12/18
	270	Neg.		

* This calculation is based on the mosquito weight of 2 mgms and the dilution required to obtain sufficient fluid to inject baby chicks. The wet chicks were injected with each mosquito suspension after trituration.

** 28/30 mosquitoes with swollen abdomens after exposure to snakes.

*** 14/17 mosquitoes found to contain WEE virus out of the total survivals in the cage 15 days after feeding on the snakes.

TABLE 2

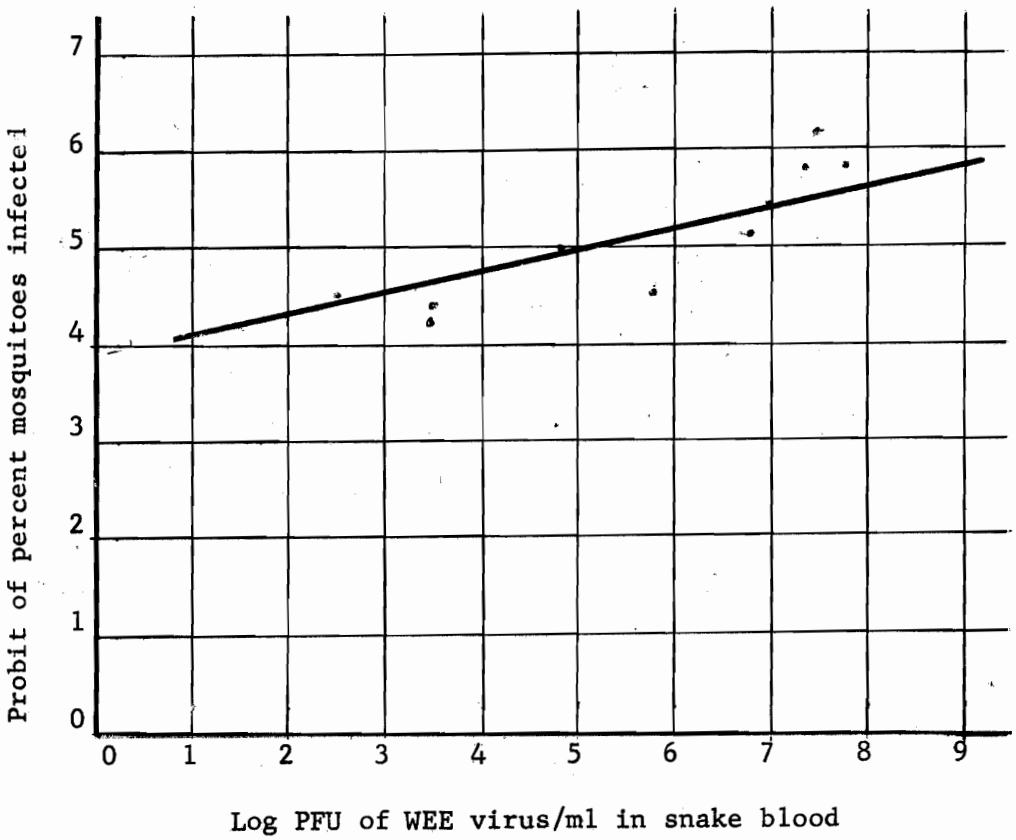
Mosquito hatching and emergence data.

Cage number	Number of pupae placed in cage.	Number of females emerging.	Number of males emerging.	Number of females taking a blood meal per total present.	Number of females surviving to end of test (15 days).
A	76	28	33	28/30*	17
B	60	23	28	27/30	16
C	63	21	31	27/30	18
D	65	19	15	28/30	17
E	51	21	16	25/30	16
F	65	14	33	19/30	15
G	67	8	17	28/30	26
H	63	17	11	24/30	19
I	65	10	9	27/30	21
J	63	28	31	23/30	18
K	68	25	26	15/30	11
L	70	30	33	26/30	18

* The female population per cage was made up to 30 using females taken from extra cages.

FIGURE 1

Relationship between the number of PFU/ml of WEE virus in the blood of snakes and the percentage of mosquitoes becoming infected



II. EXPERIMENTAL DETERMINATION OF THE NUMBER OF INFECTIVE *CULEX TARSALIS* REQUIRED TO INFECT A SNAKE.

The determination of the number of mosquitoes required to infect a snake would be of importance in discerning the possibilities of such reptiles serving as a reservoir for WEE virus.

Therefore, a preliminary study was made in which three normal snakes, Nos. 21, 39 and 70, previously found negative for virus by CEC culture assay, were placed into three infecting cages with mosquitoes which had previously engorged on an infected snake. At the time of the feeding the infected snake had an infective blood titer of 1×10^7 PFU/ml. Fourteen days after the mosquitoes had fed they were exposed to normal snakes: one mosquito was placed into each of two cages and two mosquitoes were placed into one cage. Each cage contained one snake. The following morning the snakes were removed and it was observed that all the mosquitoes had fed to repletion. The snakes were then bled periodically and the blood samples assayed for infective virus by plating on CEC monolayers, Table 3. It can be seen that snake No. 70 was converted from negative to positive after two mosquitoes had received blood meals.

III. EXPERIMENTAL INFECTION OF BATS

Bats have been suspected of being involved in the transmission of certain mosquito-borne virus diseases such as Venezuelan and Japanese B encephalitis (Corriston, 1956 and LaMotte, 1958).

TABLE 3

The ability of C. tarsalis to infect snakes as indicated by the presence of plaque forming particles in the blood of the snakes.

Snake No.	Fed on by X mosquitoes	Virus content in blood PFU/ml.			
		Prior to exposure	Four days after exposure	Nine days after exposure	Twenty-two days after exposure
21	1	0	0	0	0
39	1	0	0	0	0
70	2	0	0	8×10^5	8×10^6

Subsequently studies were initiated to determine the ability of these animals to maintain WEE virus at room and at hibernating temperatures.

The first experiment served two purposes: (1) to determine the WEE virus susceptibility of two species of bats, Plecotus townsendii (the "lump-nose" bat) a local hibernator and Taderida mexicana, (the "Mexican freetail" bat), a migratory bat, and (2) to determine the duration of the viremic state in these bats at room temperature. After finding P. townsendii was susceptible to infection and that infectious particles could be isolated from the blood, a second study was made using a larger number of these animals. In a third experiment a preliminary attempt was made to determine the ability of hibernating bats to harbor WEE virus.

Four bats were used in the first study, two Plecotus townsendii (No. 's 1P and 2P) and two Taderida mexicana (No. 's 3T and 4T). These bats were injected with LP-7 WEE virus. One of each species (No. 1P and 3T) received 0.025 ml of a virus suspension containing 1×10^3 PFU intracranially and the other of each species (No. 2P and 4T) received 1.0 ml containing 1×10^5 PFU subcutaneously. The bats were kept at room temperature and hand fed their special diet. The Mexican freetails soon learned to eat by themselves, but the lump-nosed bats remained recalcitrant. After seven days all bats except 1P, which died immediately following virus infection, were examined for the presence of virus in their blood by inoculating 6-8 day old

suckling mice with 10^{-1} and 10^{-2} dilutions of blood. The results are shown in Table 4.

The one surviving lump-nosed bat (2P) appeared ill eight days after virus injection. It was, therefore, sacrificed at this time and its organs tested for the presence of virus by inoculating tissue suspensions into suckling mice. The results of this survey are shown in Table 5.

Thirty-one days after the virus injection the Mexican freetails, No. 's 3T and 4T, were bled again and their bloods tested for virus. The negative results of these bleedings are shown in Table 4.

In the second experiment eleven P. townsendii bats were divided into four groups: members of group No. I (bats No. 5P, 6P and 7P) received 1×10^5 PFU of WEE virus subcutaneously (sc), members of group No. II (8P, 9P and 10P) received 1×10^4 PFU sc, and members of group No. III (11P, 12P and 13P) were challenged with 1×10^3 PFU of virus sc. Bats from group No. IV (14P and 15P) were challenged orally with 1×10^5 PFU of virus.

All bats were kept at room temperature. In the evening they were hand fed the diet previously described, water was kept in the cages at all times and during the day a damp gunny sack was placed over the cage and removed at night. Although the bats ate what appeared to be amounts sufficient to sustain them, there was a gradual but decided loss of weight, Table 6.

All the bats used in this study were bled by cardiac puncture prior to and periodically after challenge with virus. Results showing the presence of infectious particles in the blood of these animals as determined by suckling mouse and primary CEC plaque assay techniques are shown in Tables 7, 8 and 9 for samples collected two, four and nine days after challenge with WEE virus. A summary of these data is shown in Table 10.

The results of the first two studies seem to indicate that T. mexicana is somewhat resistant to WEE virus infection whereas P. townsendii, at least those inoculated subcutaneously, exhibited infectious virus in blood and other tissues for at least nine days. The fact that P. townsendii did so poorly in captivity, even when not challenged with virus, indicated the difficulty of meeting the proper dietary and other environmental conditions essential for survival of this species in the laboratory.

In the third study three bats (16P, 17P and 18P) were bled by cardiac puncture, a 1:10 dilution prepared as previously described and stored at -30° C until analyzed. This procedure was followed at each bleeding. After taking a zero time blood sample, the bats were injected subcutaneously with 10,000 PFU of WEE virus and placed immediately into hibernation at 6° C. Two of the bats died shortly after being placed into hibernation. The results of the periodic blood sampling of the lone surviving bat showed the blood to be

TABLE 4

The results of virus isolation attempts from Plecotus townsendii and Taderida mexicana bats seven and thirty-one days after injection with WEE virus.

Bat No.	Species	Dose LP-7 WEE Virus (PFU)	Route	Mortality of 6-8 day old mice injected with bat blood dilutions 0.025 ml/mouse ic			
				Days after virus injection			
				7		31	
				10^{-1} Dil.	10^{-2} Dil.	10^{-1} Dil.	10^{-2} Dil.
2P	Plecotus townsendii	1×10^5 in 1.0 ml	SC	4/4*	0/4	**	
3T	Taderida mexicana	1×10^3 in 0.025 ml	IC	0/4	0/4	0/4	0/4
4T	Taderida mexicana	1×10^5 in 1.0 ml	SC	0/4	0/4	0/4	0/4

* Mortality ratio

** Sacrificed sick bat 8 days after virus injection.

TABLE 5

The results of virus tissue assay. Tissues were taken from bat (P. townsendii) No. 2P eight days after injection with LP-7 virus and assayed in suckling mice.

Tissue.	Mortality of 6-8 day old mice injected with bat tissue suspensions, 0.025 ml/mouse ic.	
	10^{-1} Dil.	10^{-2} Dil.
Brain	4/4*	4/4
Liver	3/4	1/4
Brown fat	4/4	4/4
Spleen	3/4	1/4
Kidney	2/4	1/4
Heart	4/4	4/4
Blood	4/4	0/4

* Four mice died out of the four injected.

TABLE 6

Weight losses of P. townsendii captured on November 7, 1962 in a Utah cave.

Bat No.	Sex	Weight in Grams					Date Died
		Nov. 9	12	14	16	21	November
5P	F	11.3	10.2	9.5	9.0	8.4	23
6P	F	9.7	8.3	7.6	7.3		19
7P	F	11.5	11.5	9.9	9.6	8.4	24
8P	M	9.3	7.9	7.5	7.3	7.3	22
9P	F	11.4	10.6	10.2	9.3		16
10P	F	10.2	8.7	8.4	7.9		19
11P	F	10.3	9.3	8.6	8.3	8.1	26
12P	F	10.3	9.1	8.7	7.7	8.1	25
13P	F	10.0	9.0	8.5	8.1	7.8	26*
14P	M	8.9	8.4	7.9	7.4	7.0	23
15P	F	10.5	9.7	9.0	9.0	8.1	26

* Sacrificed

TABLE 7

Results of virus assay in blood of P. townsendii two days after injection with WEE virus.

Group	Bat No.	Infecting dose (PFU)	Route of inoculation	Mortality of 6-8 day old mice injected IC with 0.025 ml of bat blood samples	
				10^{-1} Dil.	10^{-2} Dil.
I	5P	1×10^5	SC	2/4	0/4
	6P	"	SC	2/4	1/4
	7P	"	SC	4/4	4/4
II	8P	1×10^4	SC	3/4	1/4
	9P	"	SC	4/4	4/4
	10P	"	SC	4/4	4/4
III	11P	1×10^3	SC	2/4	0/4
	12P	"	SC	1/4	0/4
	13P	"	SC	4/4	4/4
IV	14P	1×10^5	ORAL	0/4	0/4
	15P	"	ORAL	0/4	0/4

TABLE 8

Results of virus assay in blood of P. townsendii four days after injection with WEE virus.

Bat No.	Mortality of 6-8 day old mice injected IC with 0.025 ml bat blood dilutions.		Mortality of 6-8 day old mice injected IC with 0.025 ml mouse brain suspensions. *	
	10^{-1} Dil.	10^{-2} Dil.	10^{-1} Dil.	10^{-2} Dil.
5P	2/4	0/4		
6P	2/4	1/4		
7P	4/4	4/4	4/4	4/4
8P	3/4	1/4		
9P	4/4	4/4	4/4	4/4
10P	4/4	4/4		
11P	2/4	0/4		
12P	1/4	0/4		
13P	4/4	4/4		
14P	0/4	0/4		
15P	0/5	0/4		

* These suspensions were made from the brains of mice dying as a result of injection with the bat blood dilutions.

TABLE 9

Virus assay of blood specimens taken nine days after inoculation of P. townsendii with WEE virus.

Bat No.	Mortality of 6-8 day old mice injected IC with 0.025 ml bat blood dilutions.					Virus titer on chick embryo cell monolayer cultures in PFU/ml.
	10^{-1} Dil.	10^{-2} Dil.	10^{-3} Dil.	10^{-4} Dil.	10^{-5} Dil.	
5P	4/4	4/4				
6P	4/4	4/4				
7P	4/4	4/4	3/4	2/4	0/4	2.8×10^5
8P	4/4	4/4	2/4	2/4	2/4	6.4×10^4
9P*						
10P*						
11P	4/4	1/4				
12P	4/4	4/4				
13P	4/4	4/4	4/4	4/4	2/4	4.1×10^5
14P	0/4	0/4				
15P	0/4	0/4				

* Died from previous bleeding.

TABLE 10

Summary of the virus assays in the blood of P. townsendii with WEE virus two, four and nine days after injection.

Bat No.	Dose LP-7 virus (PFU's)	Route	Mortality of 6-8 day old mice injected with bat blood dilutions 0.025 ml/mouse IC									
			Prior to virus injection		Days after virus injection							
					2		4		9			
			10^{-1} (Dil.)	10^{-2} (Dil.)	10^{-1} (Dil.)	10^{-2} (Dil.)	10^{-1} (Dil.)	10^{-2} (Dil.)	10^{-1} (Dil.)	10^{-2} (Dil.)	10^{-3} (Dil.)	10^{-4} (Dil.)	10^{-5} (Dil.)
5P	1×10^5	SC	0/4	0/4	2/4	0/4	4/4	1/4	4/4	4/4			
6P	"	SC	0/4	0/4	2/4	0/4	4/4	4/4	4/4	4/4			
7P	"	SC	0/4	0/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4	2/4	0/4
8P	1×10^4	SC	0/4	0/4	3/4	1/4	3/4	3/4	4/4	4/4	2/4	2/4	2/4
9P	"	SC	0/4	0/4	4/4	4/4	4/4	2/4					
10P	"	SC	0/4	0/4	4/4	4/4	4/4	4/4					
11P	1×10^3	SC	0/4	0/4	2/4	0/4	4/4	1/4	4/4	1/4			
12P	"	SC	0/4	0/4	1/4	0/4	4/4	4/4	4/4	4/4			
13P	"	SC	0/4	0/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	2/4
14P	1×10^5	ORAL	0/4	0/4	0/4	0/4	0/4	1/4	0/4	0/4			
15P	"	ORAL	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4			

negative for virus after 12 days of hibernation, but after 36 days of hibernation a viremia of 2.3×10^3 PFU/ml of blood was detected. An analysis for the presence of virus in the tissues of this bat is shown in Table 11. As can be seen, virus was isolated from the blood of this animal 36 days after it was injected and placed into hibernation, virus was also isolated from the tissues of this same animal on the day it died or the 45th day of hibernation.

IV. RESULTS OF VIRUS ISOLATION AND ANTIBODY DETECTION ATTEMPTS FROM WILD CAUGHT SNAKES AND BATS

In 1961 forty-one garter snakes were caught in marshy areas bordering the Great Salt Lake. No virus was isolated from the blood of any of these reptiles using wet chick, suckling mice and CEC culture isolation techniques. Attempts to detect WEE antibody in these animals using mouse neutralization and plaque reduction tests also failed.

One of 51 snakes captured between March 30 and April 20, 1962 in the vicinity of the Newstate Duck Club possessed circulating virus in February, 1963. A subsequent plaque reduction test using specific WEE antiserum proved it to be WEE virus. When this snake was bled on May 5, 1962 and tested for virus by wet chick inoculation, approximately one month after capture, one of the three chicks inoculated died; however, no follow-up tests were run since the death was attributed to drowning. The infected snake was tagged at the time of the first

TABLE 11

Occurrence of WEE virus in tissues of a P. townsendii bat dying 45 days after hibernation at 6° C. *

Tissue	Mortality of 6-8 day old mice injected with bat tissue suspensions in Earles complete growth media 0.025 ml/mouse IC	
	10^{-1} Dil.	10^{-2} Dil.
Brain	0/4	0/4
Liver	2/4	1/4
Brown Fat	4/4	4/4
Spleen	4/4	3/4
Kidney	0/4	0/4
Heart	3/4	1/4

* The bat was injected with 10^4 PFU of WEE virus.

bleeding and kept in a screened tank at room temperature along with other previously determined normal snakes until virus was isolated on the second attempt. It is believed this is the first time WEE virus has been isolated from a reptile in nature. Attempts to detect WEE neutralizing antibody in the group of animals captured in 1962 also failed.

Of the twenty bats tested for virus and WEE neutralizing antibody during the summer and fall of 1962 using methods previously described, none were found to have infectious virus or specific antibody in their bloods.

DISCUSSION

The ability of hibernating garter snakes to carry WEE virus for extended periods of time is now well known (Gebhardt and Hill, 1960). Snakes infected experimentally in the fall are capable of infecting mosquitoes the following spring (Thomas and Eklund, 1962). The infected mosquitoes could thus initiate the summertime mosquito-bird-mosquito endemic and/or epidemic cycle of WEE found in nature. The fact that both the viremia in snakes (Gebhardt et al., 1960 and Thomas et al., 1962) and the build up of C. tarsalis populations in the spring or early summer (Rush et al., 1958 and Bennington et al., 1958) are temperature dependent allows for much variation in environmental conditions before a cycle of this type can be broken. When temperatures are cold in the spring the duration of viremias in snakes are prolonged as well as the build up of C. tarsalis populations and vice-versa.

The unusual capability of garter snakes to overwinter WEE virus and to exhibit lengthy viremias in the spring has made them prime suspects in the natural history of this disease. However, the isolation of WEE virus from this animal in nature was never accomplished until now, and although only one out of 92 snakes tested so far was found to harbor WEE virus, the conditions of the isolation were significant and interesting.

First of all, since the single snake was captured between March and April 22, 1962 prior to the emergence of C. tarsalis from hibernation in this area, the possibility is eliminated that this snake became infected by C. tarsalis during the same spring. Conceivably, the only possible remaining explanation, at this time, is that the snake became infected prior to hibernation, thus demonstrating its ability to overwinter virus.

Secondly, at the time of the bleeding the blood titer of the snake was in excess of the number of virus particles capable of infecting over forty per cent of the mosquitoes feeding on it as calculated from the experimental evidence presented in this thesis.

Thirdly, since the cage the snake was housed in was screened and contained only normal snakes, and since in addition infected snakes have never been found capable of passing the virus to other snakes without a vector, it is assumed that the snake was infected at the time of capture.

A fourth point should also be explored concerning the possibility that this snake was negative for circulating virus at the time of the first bleeding and subsequently became positive during the eleven months it was held in captivity. Other researchers have found snakes capable of converting from negative to positive for the presence of virus when coming out of hibernation (Gebhardt et al., 1960 and Thomas et al., 1962), but in this instance a snake held in captivity

at room temperatures became positive for circulating virus. The fact that viremias in these animals seems to be of a cyclic nature leads one to the conclusion that testing these animals at one particular time of the year is not sufficient for detection of circulating virus. Therefore, further studies with the aim of determining more efficient means of detecting virus in snakes caught in the wild should possibly be directed toward the site of localization of virus in the tissues of these animals.

In conclusion, since isolation of virus from a single snake caught in nature is not decisive evidence for these animals being the overwintering reservoir, it is highly suggestive and stimulative for continued research in this area of study.

In examining the data obtained in the snake to mosquito transmission experiment it was found that in a mosquito such as C. tarsalis, which imbibes as little as 0.003 ml of blood during a meal, as few as 10 PFU of virus can infect 27 per cent of the insects ingesting blood meals. When snakes possess titers of $10^{5.2}$ infectious particles per ml, 50 per cent of the mosquitoes obtaining blood meals become infected. Titers in excess of 10^{7} PFU per ml will infect from 79 to 87 per cent of the mosquitoes. In general, even though much variation in the results was found, there seems to be a definite relationship between increasing virus content in snake blood and the increasing ability to infect mosquitoes feeding on this blood. Every snake showing

the presence of infectious particles in its blood was capable of infecting some of the mosquitoes feeding on it.

The duration of viremia in these animals kept at room temperatures was highly variable. One snake was positive two days after injection and then negative two days later. The other three snakes, showing much higher peak titers than the previously mentioned snake, had viremias enduring for at least seven days and probably longer. These three snakes were all negative approximately ten days after injection. Of interest is the fact that the snake infected by the bite of two mosquitoes developed a viremia more slowly than the injected snakes and also was still positive 22 days after exposure to the mosquitoes.

It would seem, concluding from the evidence presented, that snake blood can serve as a highly efficient source of WEE virus for mosquitoes. When injected with lower dosages of virus, as from a mosquito, the resulting viremia could possibly be prolonged, thus assuring many days of mosquito infectibility for these reptiles.

In a study to determine the number of mosquitoes required to infect a snake it was found that while one was not, two were sufficient. A more extensive study using greater numbers of animals will have to be done in order to substantiate these preliminary findings. In any event it now seems that relatively few mosquitoes are needed to infect a snake and that one snake is capable of infecting a large

percentage of the mosquitoes feeding on it. If the assumption is made that a relatively small number of infected mosquitoes are needed to start and maintain an endemic cycle of WEE virus; then the snake seems to fulfill, at least in the laboratory, the criteria for serving as a reservoir for WEE virus.

Much more field work is needed in the ecology of this virus especially in its relationship to snakes before a definite theory can be formulated on the importance of these animals in maintaining WEE virus in nature. Therefore, additional studies as follows will be conducted.

In addition to testing more snakes periodically for virus, caged sentinel snakes will be housed in different areas where large C. tarsalis populations are existent and where previous experience and knowledge has lead one to believe virus is present in animals in the area. Mosquito attack rates on the sentinel snakes will be determined and periodic blood samples taken for virus assay. These data in association with the preliminary results presented in this thesis, will give greater insight into the possibilities of snake-mosquito-snake virus transmission under natural conditions. When this field problem has been solved one might be able to predict future encephalitis outbreaks by assaying snakes for virus in a particular area at a particular time.

In addition to the previously planned studies, further investigations into the viremia cycling phenomena found in these snakes will

be made. Snakes will be infected in July and August both by injection and by allowing various numbers of infected mosquitoes to feed upon them. A group of these snakes will be hibernated while still exhibiting circulating virus and another group will be hibernated after the viremia has subsided. Periodic blood and tissue sampling will then be conducted over a period of eight months. Some knowledge should be gained from this study on the capabilities of hibernating snakes infected in mid-summer to develop viremias the following spring.

In the bat infecting studies, evidence has been presented indicating that certain of these species are capable of becoming infected and circulating virus in their blood for extended periods of time under hibernating conditions. Other species are not. No mechanisms for this difference can be postulated as yet, however, the fact that P. townsendii is a hibernating bat while T. mexicana is primarily a migratory bat might be important. Although there is a paucity of data available at this time, hypothetically these animals could still be important either as a hibernating reservoir for virus or as a migratory means of bringing virus into an area.

Future studies planned for bats might aid in formulating a theory regarding the possible role of these animals in the ecology of this virus in nature.

SUMMARY

The experimental and field evidence presented in this thesis relative to the possibility that snakes are important in the over-wintering mechanism of WEE virus in nature are as follows:

1. Virus titers in snakes were found to be sufficiently high to infect a significant percentage of mosquitoes feeding on them. The ID_{50} for a mosquito was found to be approximately $10^{5.2}$ PFU/ml.
2. The duration of viremia in these animals was considerably longer, even at room temperature, than has been found in most other animals.
3. Under the conditions of the experiments, two infective mosquitoes were sufficient to infect certain snakes.
4. Virus was isolated from at least one snake captured in nature during the spring, when recent infection by C. tarsalis was impossible.
5. Possible evidence for a cycling phenomena of WEE virus in the blood of snakes kept at room temperature was discussed.

In preliminary studies involving experimental infection of bats with WEE virus it was found that under hibernating conditions, one species of bat, P. townsendii, would maintain WEE virus in the circulating blood for at least 36 days and in tissues for at least 45 days.

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POSSIBLE OVERWINTERING RESERVOIRS AND EXPERIMENTAL EPIDEMIOLOGY OF WESTERN EQUINE ENCEPHALITIS VIRUS

by

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ABSTRACT

Snakes infected with Western equine encephalitis virus were exposed periodically to groups of normal Culex tarsalis mosquitoes. The relationship between the number of plaque forming units (PFU) of virus circulating in snake blood and the subsequent ability of this number of virus particles to infect mosquitoes taking blood meals was explored. Data were also obtained on the magnitude and temporal characteristics of viremia in these reptiles kept at room temperature.

Although the data collected in the mosquito infecting experiment were variable, they indicated the mid-infective virus dose (ID_{50}) for mosquitoes engorging on infected snake blood was approximately $10^{5.2}$ PFU/ml of blood. The data also showed that the more virus particles taken in by a mosquito the greater was the liklihood of its becoming infected.

The persistence and magnitude of the viremia in the snakes was also variable. One snake was positive for circulating virus two days after injection and then negative two days later while the other three snakes exhibited viremias from approximately seven to ten days. One snake infected by the bite of two mosquitoes developed a viremia more slowly than the injected snakes and was still positive 22 days after exposure to the mosquitoes.

The virus titers in the blood of the snakes ranged from $10^{2.4}$ PFU/ml for the snake showing the shortest viremia to $10^{7.8}$ PFU/ml for a snake still positive seven days after challenge. The average peak titers of three snakes was over 10^{7} PFU/ml.

By exposing normal snakes to infective mosquitoes it was found that at least two mosquitoes were required to infect a snake.

In field studies conducted along with the experimental studies, WEE virus was isolated from one of 51 snakes captured between March 30 and April 20, 1962. At the time of capture C. tarsalis had not emerged from hibernation, thus the assumption was made that the snake was infected prior to hibernation. This is the first reported isolation of WEE virus from a reptile.

In association with the isolation of virus from the snake more evidence was presented for the hypothesis that viremias in these reptiles are of a cyclic nature.

In preliminary studies on experimental infection of bats with WEE virus, it was found that under hibernating conditions, one species of bat, Plecotus townsendii, maintained virus in the circulating blood for at least 36 days and in tissues for at least 45 days. This same species of bat kept at room temperatures exhibited virus in the blood for at least nine days.

In conclusion, the evidence presented is highly suggestive for further investigations on the possibilities of reptiles and/or bats serving as overwintering reservoirs for WEE virus in nature.